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OSTASH B.O.[1], YUSHCHUK O.S.[1], KOSHLA O.T.[1], REBETS Y.[4], OSTASH I.S.[1], SEHIN Y.V.[1], BUSCHE T.[2], KALINOWSKI J.[2], MUTH G.[3], FEDORENKO V.O.[1]✉

[1] Ivan Franko National University of Lviv,
Ukraine, 79005, Lviv, Hrushevskoho, 4
[2] Center for Biotechnology, Bielefeld University,
Germany, Bielefeld
[3] Interfaculty Institute of Microbiology and Infection Medicine, Tubingen University,
Germany
[4] Harvard Medical School,
United States of America, MA 02215, Boston
✉ *v_fedorenko@franko.lviv.ua, +38(032) 239-44-07, +38(032) 239-44-75*

ELUCIDATION OF THE GENETIC MECHANISMS CONTRIBUTING TO MOENOMYCIN RESISTANCE IN ACTINOBACTERIA

Aim. Moenomycins are phosphoglycolipid antibiotics produced almost exclusively by representatives of genus *Streptomyces*. These antibiotics directly inhibit peptidoglycan glycosyltransferases and are extremely active against cocci. Here we studied how antibiotic-producing actinobacteria protect themselves from toxic action of moenomycins. ***Methods***. Microbiological and molecular genetic approaches were combined to reveal intrinsic levels and distribution of moenomycin resistance across actinobacteria genera, and to pinpoint genes contributing to moenomycin resistance in model strain *Streptomyces coelicolor* M145. ***Results***. Out of 51 actinobacterial species (90 % of which *Streptomyces*) being tested, only *Streptomyces albus* J1074 turned out to be highly susceptible to moenomycin A, although resistant variants can be facilely raised. Several classes of mutations increased level of susceptibility of *S. coelicolor* to moenomycin, although in no case the latter was equal to what we observed in J1074 strain. ***Conclusions***. Moenomycin resistance is widespread across actinobacteria, and it most likely is caused by a combination factors, such as richly decorated cell wall and organization of divisome apparatus. It is possible that moenomycin resistance mechanisms operating in actinobacteria and pathogenic cocci are different.
Keywords: moenomycin, antibiotic resistance, peptidoglycan.

Moenomycin A (MmA; Fig. 1) represents small family of phosphoglycolipid natural products that possess good spectrum of activity and a unique mode of action against bacteria [1]. MmA is not used clinically because of poor pharmacokinetics, which for more than 30 years motivated the search for better tools to manipulate its structure and generate improved analogs. Recently, total synthesis of MmA has been realized [2], and genes for moenomycin biosynthesis (*moe*) have been cloned, advancing our ability to search chemical space around phosphoglycolipid scaffold. Furthermore, structural details of interaction between moenomycins and their target, peptidoglycan glycosyltransferases (PGTs; or transglycosylases), have been illuminated [3]. Despite the progress in understanding chemistry and biology of moenomycins, it is not known how MmA-producing bacteria avoid its toxic action [4, 5]. Knowledge of such mechanism(s) would help understand and slow down the spread of MmA resistance among pathogens. Little attention to this problem in case of glycopeptide antibiotics has led to dramatic rise of vancomycin-resistant bacteria, contributing to a great extent to current crisis in antibiotic therapy [6]. In this work we explored distribution of MmA susceptible and resistant species, as well as morphological and genetic factors that might contribute to the resistance. We show here that a number of factors contribute to MmA resistance in *Streptomyces albus* J1074 and *S. coelicolor* M145, such as spectrum of expressed PGT, divisome (*mreB*) and wall teichoic acid genes.

Materials and methods

Actinobacteria used in this work are stored in the collection of microorganisms-producers of antibiotics at the Department of Genetics and Biotechnology of the University. The studied strains included all known moenomycin producers, model

strains (*S. coelicolor* M145, *S. lividans* 1326, *S. albus* J1074), representatives of genera *Micromonospora*, *Amycolatopsis*, *Saccharopolyspora*, *Actinomadura*, *Pseudonocardia* and *Actinoplanes*. Full list of strains is available from authors upon request. *Escherichia coli* strains ET12567 (pUB307) and DH5α were used for intergeneric conjugations and routine cloning, respectively.

Cosmids used for gene disruption in *S. coelicolor* M145 (kindly provided by Prof. P. Dyson, University of Swansea, UK) are as follows: K7.1.E06 (insertion of Tn5062 into *sco5039*, PGT domain); SCE6.1.G10 (*sco2897*, PGT domain); h24-2.B01 (*sco3901*, PGT domain); SCE50.1.H07 (*sco2983*, similar to TagF, teichoic acid glycosyltransferase); C123.2.F09 (*sco2590*, similar to TagF, teichoic acid glycosyltransferase); SC4C2.1.B08 (*sco7671*, homolog of *S. grisesus srsA* for phenolic lipid synthesis). Expression vectors pKC1139E, pTES and pIJ6902 [7] were used to clone genes as XbaI-EcoRI fragments into respective sites of the vector. All plasmids were verified via sequencing. Sequences of the primers are available from authors upon request. *E. coli* strains were grown at 37°C in LB, 2×TY or TB for routine applications. *Streptomyces* strains were grown at 30°C. Solid oatmeal medium (OM [7]) was used to plate conjugation mixtures. For genomic and plasmid DNA isolation, streptomycetes were grown in TSB for 48–60 h.

Antibiotic resistance was assayed on solid Bennett and MM media; *S. coelicolor* protoplasts were regenerated on solid R2YE as described in [7]. Pure MmA was kindly provided by Prof. D. Kahne (Harvard University).

BLAST search tools (on the server of National Center for Biotechnology Information (NCBI), Bethesda, MD) were used for identify genes of interest in the genomes of *Streptomyces*. The CDD search engine (BLAST server) and set of programs (HHPred, Pfam, TMHMM, MUSCLE) on the HHSuite toolkit were used (https://toolkit.tuebingen.mpg.de/#/).

Standard procedures were employed to isolate, construct and analyze recombinant DNA and RNA. *E. coli – Streptomyces* conjugation and protoplast transformation were done as described [7]. In protoplast regeneration experiments, titer of regenerants on antibiotic-supplemented plates was calculated taking into account the number of unlyzed mycelia (determined plating of protoplasts resuspended in 0.01 % SDS). Primary analysis of antibiotic resistance has been carried out with the disc diffusion method. MmA resistance was also analyzed by the titration of actinomycete spore suspensions on plates containing increasing MmA concentrations. We used commercially available discs or prepared them from fresh antibiotic stocks and Whatman discs (∅5 mm).

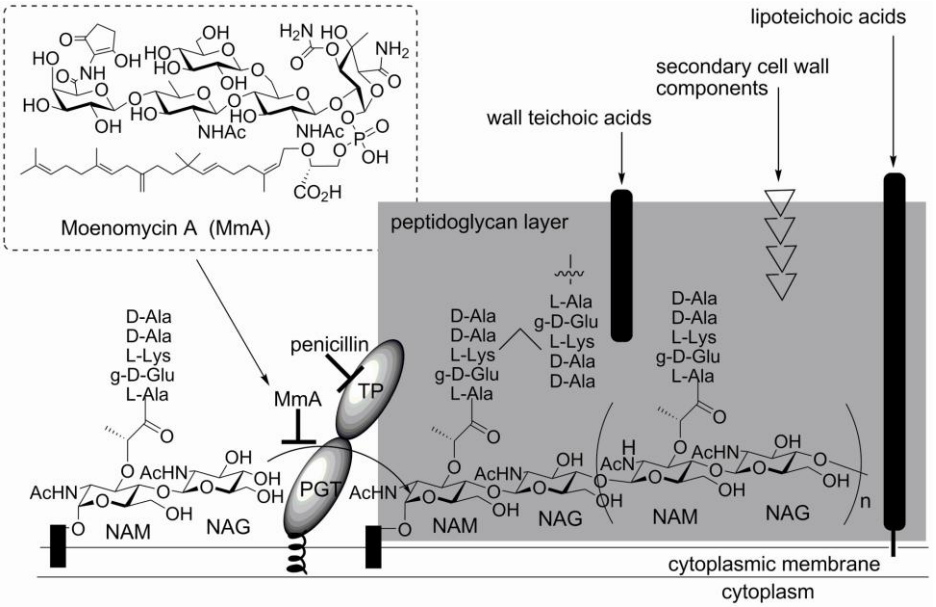

Fig. 1. Moenomycin A (MmA) and extracellular steps of bacterial peptidoglycan biosynthesis. Sites of MmA and penicillin inhibition are indicated. Penicillin binding proteins (PBPs) are shown with both peptidoglycan glycosyltransferase (PGT) and transpeptidase (TP) domains. A part of peptidoglycan layer is shown as a grey shade where other cell wall components (teichoic acids etc) are embedded. Lipid part of Lipid II is shown as black rectangle embedded into cytoplasmic membrane.

Gene knockouts in *S. coelicolor* with the help of transposon cosmids were carried out essentially as described in [8]. Three representative Am^rKm^s clones for each knockout were used for analysis of the morphology and antibiotic resistance. All mutations were PCR-verified in two rounds. First, the absence of certain wild type gene in the knockout strain has been verified as a failure to amplify it with primers to the ends of the gene. Second, replacement of intact allele with mutated one was checked through a combination of transposon-specific primer (EZR1 or EZL2) and a *sco*-specific primer (5′- or 3′-terminal). In all cases we observed the expected pattern of PCR bands.

For electron microscopy biomass was grown in TSB for 3 days. The 2 ml sample of the 3-day culture was spun down, washed with water three times and resuspended in a fixative made of 2 % glutaraldehyde in 0.2 M cacodylate buffer at pH 7.4. The samples were dehydrated with successive solutions containing increasing concentration of ethanol and submitted to the Harvard Medical School Electron Microscopy (EM) Facility for processing.

Results and discussion

In this work we pursued several research directions. First, it was important to understand whether MmA resistance is characteristic of moenomycin-producing strains, or it is readily present also in MmA nonproducers and non-streptomycete species. Second, using spontaneous MmA resistant (MmA^r) variants of *S. albus*, we explored scenarios that might underline the MmA resistance. Besides peptidoglycan, the Grampositive cell wall of *Streptomyces* is decorated with a number of components (see Fig. 1), which might contribute to MmA resistance. Therefore, in a third topic we focused on analysis of genes for cell wall biogenesis, membrane lipids and cytokinesis as possible MmA resistance targets. Finally, we studied the role of several transporter genes in MmA resistance. Below we describe our results along these four research lines and discuss the most promising future research directions.

MmA resistance is widespread across actinobacteria. We wanted to study MmA resistance in a phylogenetically diverse group, therefore non-*Streptomyces* strains were included. Second, *Streptomyces* strains picked for analysis cover all known moenomycin producers as well as one mutant with abolished MmA synthesis and several model strains, whose genomes are available (see Methods). The latter will provide a rich genetic background for further studies on MmA resistance. The other streptomycetes were chosen because peculiarities of their growth or susceptibility to other antibiotics that may impact MmA resistance. For example, *S. hawaiinesis*, *S. sioyaensis* and *S. nogalater* are more sensitive to beta-lactams than other *Streptomyces*, which may impact MmA susceptibility. *S. venezuelae* exhibits disperse, single-celled mode of growth. *S. avermitilis* produces no rodlins, small hydrophobic protein that covers aerial hyphae. It was interesting to compare its MmA resistance profile to other strains possessing the rodlet layer. To this end, we revealed that only *S. albus* J1074 exhibited significant susceptibility to MmA. In comparison with MmA producer *S. ghanaensis* or *S. coelicolor*, its survival rate at the lowest and the highest MmA concentrations tested (1 and 100 mcg/ml) dropped more than 4 and 6 orders of magnitude, respectively (Fig. 2). All other actinomycetes were resistant to MmA, showing little or no connection of the studied trait to morphology or phylogeny of the strain.

Spontaneous MmA^r resistant mutants of *S. albus* J1074: generation and initial studies. After 5 days of growth, 6 MmA^r *S. albus* colonies were observed in a zone of lysis around disc soaked into MmA. After three passages under non-selective conditions, their survival in presence of MmA has been determined. We finally identified a mutant, referred to as *S. albus* R1-100, that stably confirmed its Mm^r phenotype and showed 44±3 % survival in presence of 100 mcg/ml of the antibiotic (compare to 10^{-5} % for J1074, Fig. 2). Its resistance to other antibiotics remained unchanged. There were no differences in growth rate or sporulation of the R1-100 strain in comparison to parent J1074. Using electron microscopy (Fig. 3), we attempted to compare the thickness of cell walls of *S. albus* strains J1074 and R1-100, *S. coelicolor* and *S. ghanaensis*. The cell wall thickness was within 0,017–0,026 μm range for the studied strains (300 sections were analyzed in each case, an example is shown on Fig. 3). No statistically significant differences in the measured parameter were detected between the strains. We did observe that R1-100 began to produce copiously a yellow pigment, which seems to be produced by J1074, too, albeit at a much lower level. We note here that production of certain primary and secondary metabolites, such as lipid II and carotenoids could confer bacteria to MmA resistance [3], and this might also be the case for R1-100. However, our numerous attempts to find suita-

ble conditions of extraction and TLC separation of the pigment haven't met with success. Resistance to cell wall-active antibiotics is often modulated by the structure of cell wall polymers other than peptidoglycan, such as teichoic acids. We analyzed the structure of these cell wall polymers in R1-100 and revealed that they are identical to that of J1074 [9], qualitatively and quantitatively.

Cell wall as a MmA resistance determinant. If the PGTs involved in *S. coelicolor* M145 cell wall biosynthesis are intrinsically resistant to MmA, then both cells and protoplasts of M145 should display approximately the same level of resistance. The protoplasts of M145 strain have been prepared and regenerated on agar plates in presence of either MmA or penicillin G (100 mcg/ml in each case). MmA and penicillin G reduced the protoplast survival to 0.6±0.4 % and 20±4 %, respectively, as compared to untreated protoplasts (100 %; average protoplasts titer was 6.1×10^7 ml^{-1}). Thus, our data point to the fact that cell wall does contribute to MmA resistance of *S. coelicolor* and, probably, other streptomycetes. Yet, the level of *S. coelicolor* protoplast survival reduction as compared to cells (around 100-fold) is not as dramatic as we observed for *S. albus* J1074 and R1-100 (over 10000-fold). Hence, cell wall composition alone could not explain high level MmA resistance in *S. coelicolor*.

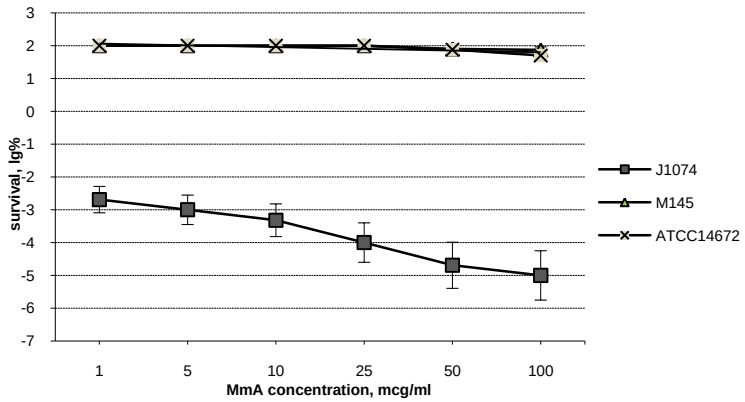

Fig. 2. Survival curves of strains *S. coelicolor* M145, *S. ghanaensis* ATCC14672, *S. albus* J1074 in presence of increasing amounts of MmA.

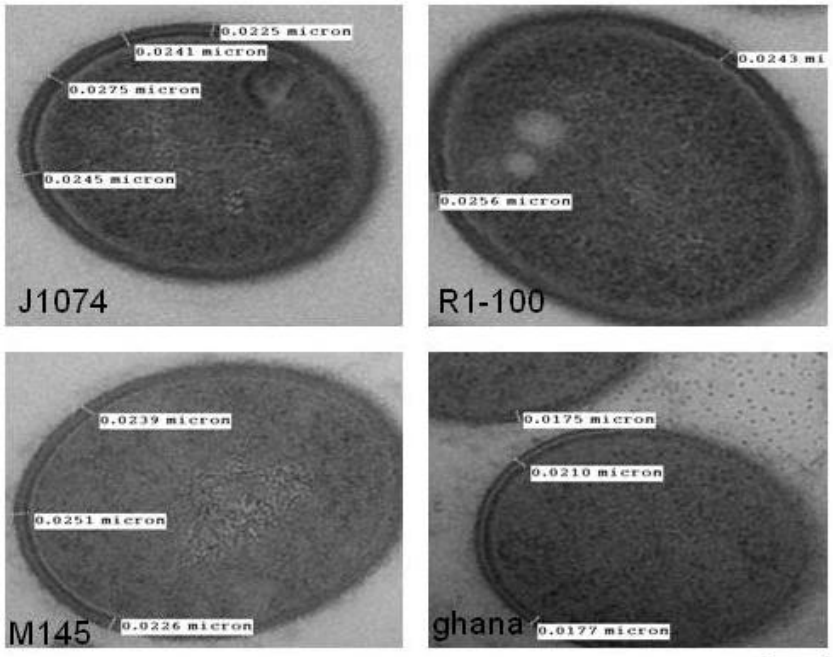

Fig. 3. EM images of hyphal sections of *S. albus* strains J1074 and R1-100, *S. coelicolor* M145 (M145) and *S. ghanaensis* ATCC14672 (ghana). Scale bar under the photos, 100 nm.

Dissecting the contribution of genes for membrane, cell wall biogenesis and cytokinesis in MmA resistance. Protoplasts studies suggested that streptomycete PGTs carry no special amino acid substitutions that would relieve their inhibition with MmA.This notion has been further reinforced when we inspected multiple alignment of amino acid sequences of PGTs from *S. coelicolor* and *S. ghanaensis*: no known amino acid substitutions leading to low affinity towards MmA [10, 11] can be observed in PGTs of aforementioned species. *S. albus* PGTs are almost identical to other PGTs being compared, and the observed variability is common to MmA-sensitive PGTs from *E. coli* or cocci. Hence, MmA resistance of *S. coelicolor* and *S. ghanaensis* is not likely to result from low-affinity PGTs. Recently RodA enzymes in *Bacillus* and *Escherichia* were shown to possess PGT activity that is unsusceptible to MmA [12]. We identified RodA orthologs in *S. coelicolor* M145 (Sco2607) and *S. albus* J1074 (Xnr4338). Through analysis of our own and publicly available transcriptomes, we found no differences in *rodA* expression in *S. coelicolor* and *S. albus*.

Next we profiled MmA resistance in a set of *S. coelicolor* strains deficient in one of the genes involved in cell wall biogenesis, membrane lipids production and division. First, we generated, using transposon cosmids, a set of *S. coelicolor* mutants deficient in genes for peptidoglycan (PGT genes *sco2897*, *sco3901*, *sco5039*), teichoic acids (*sco2590*, *sco2983*) and putative phenolic lipids (*sco7671*) biosynthesis. All generated mutants did not differ from the parent strain in growth rate or sporulation. In terms of antibiotic resistance, *sco3901* mutant was more susceptible to MmA (20±3 % survival rate at 100 mcg/ml of antibiotic compared to 87±5% for wild type) and *sco7671* mutant was 25 times less resistant to penicillin G at 100 mcg/ml (4±1 % vs 97±5 %). We also overexpressed PGT genes in *S. coelicolor* in hope that increased quantities of MmA target proteins might lead to increases in MmA susceptibility; however no changes in antibiotic resistance have been detected (data not shown). Finally, we examined following *S. coelicolor* strains disrupted in cell wall biosynthesis (*pbp2*, *sco2997* (*tagF*-like), *sco2578* (*tagV*-like)) and cytokinesis (*mreB*, *mreC*) genes described by us in previous works [13]. As compared to M145, *mreB*, *mreC* and *sco2997* mutants were more susceptible to MmA after 48 h of growth in presence of antibiotic disc, however later on (72 h) halo of growth inhibition disappeared (Fig. 4). In all, it appears that impairment of cell wall structure facilitates the access of MmA to PGTs; however, none of these mutations lead to a level of MmA susceptibility observed for *S. albus*. Therefore, as-yet-unstudied MmA-resistant PGTs operate in *Streptomyces*.

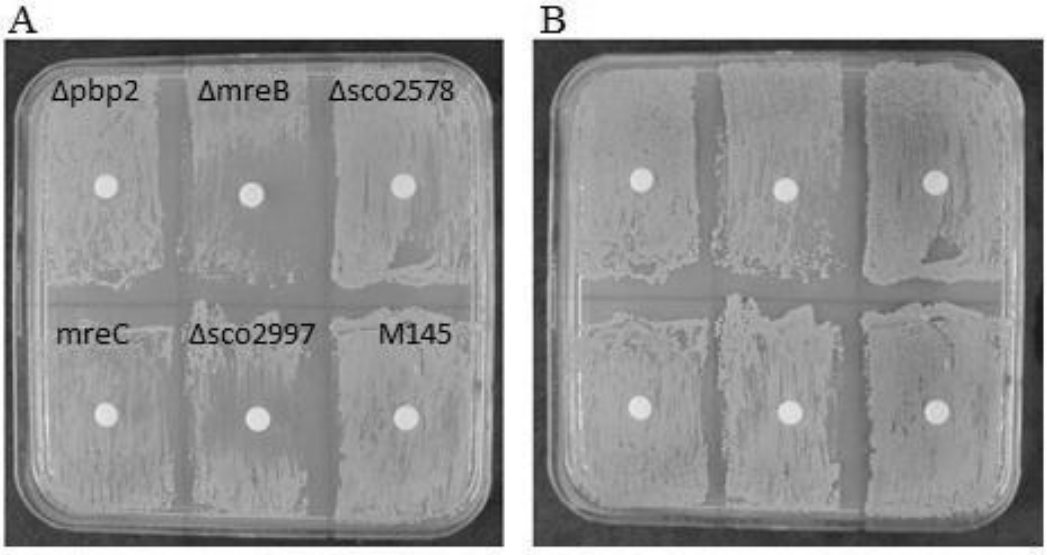

Fig. 4. MmA resistance among selected *S. coelicolor* mutants after 48 (**A**) and 72 (**B**) h of growth on TSA. 10^7 spores were seeded to obtain patches of lawns where discs impregnated with 50 mcg of MmA were stacked upon.

Transporter genes in moenomycin resistance. Export of peptidoglycan precursor Lipid II (see Fig. 1) requires dedicated transporter proteins [14]. Such transporters may also be involved in active extrusion of MmA [3]. We identified, *in silico*, two presumable Lipid II flippases in *S. ghanaensis* genome, *SSFG_03627* and *SSFG_01411*, and introduced them (on ϕC31-based integrative vector pTES) into *S. albus* J1074. As control cases, we introduced into J1074 two transporter genes from landomycin A producer *S. cyanogenus* S136: *lanJ* (plasmid pOOB104a) and *lanJ* paralog *scy3375* (pMO38a). Two latter genes are not related to export of lipid-linked polysaccharide, and increased MmA resistance of pOOB104a- or pMO38a-borne J1074 would be construed as a result of nonspecific MmA export. None of the generated strains had distinct MmA resistance profile.

Conclusions

MmA resistance is widespread in actinobacteria, and is not caused by the intrinsic low affinity of typical PGTs towards the antibiotic. Chemical composition of streptomycete cell wall, particularly presence of teichoic acids, contributes to the high level MmA resistance, although cell wall itself is not a key resistance determinant. Clearly, cell wall lesions create temporary perforations allowing PGT targeting by MmA. Principal mechanism of MmA resistance in *Streptomyces* remains elusive. It is important to undertake broader study of RodA homologs in actinobacteria and to carry out deeper physiological and genomic analysis of *S. albus* R1-100 strain.

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ОСТАШ Б.О.[1], ЮЩУК О.С.[1], КОШЛА О.Т.[1], РЕБЕЦЬ Ю.В.[4], ОСТАШ І.С.[1], СЕГІН Ю.В.[1], БУШЕ Т.[2], КАЛІНОВСКІ Й.[2], МЮТ Г.[3], ФЕДОРЕНКО В.О.[1]
[1] Львівський національний університет ім. І. Франка,
Україна, 79005, м. Львів, вул. Грушевського, 4
[2] Центр Біотехнології , університет Білефельда,
ФРН, Білефельд
[3] Інститут мікробіології та медицини, Тюбінгенський університет,
ФРН
[4] Гарвардська медична школа,
США, МА 02215, Бостон

ВИВЧЕННЯ ГЕНЕТИЧНИХ МЕХАНІЗМІВ СТІЙКОСТІ ДО МОЕНОМІЦИНУ В АКТИНОБАКТЕРІЙ

Мета. Дослідити генетичні механізми стійкості до моеноміцину А (МмА) в актинобактерій. ***Методи***. Використано мікробіологічні та молекулярно-генетичні підходи, та модельні штами *Streptomyces coelicolor* M145 й *S. albus* J1074 для виявлення поширення досліджуваної ознаки, а також роль компонентів клітинної стінки на рівень резистентності. ***Результати***. Серед 51 дослідженого штаму актинобактерій виявлено, що тільки *S. albus* J1074 високочутливий до МмА. Отримано мутанти J1074 стійкі до МмА. Рівень стійкості останніх не визначається потовщенням клітинної стінки. Один із Мм^r мутантів характеризується підвищеною продукцією наразі неідентифікованих забарвлених метаболітів. Хімічний склад клітинної стінки впливає на рівень стійкості до МмА, але не є визначальним фактором. Пошкодження синтезу тейхоєвих кислот мало найбільший вплив на рівень стійкості до МмА; другий за величиною вплив мали гени дивізомного комплексу (*mreB*). ***Висновки***. Стійкість до МмА в стрептоміцетів визначається поєднанням особливостей хімічного складу клітинної стінки та, імовірно, експресії наразі невиявлених низькоафінних до МмА пептидогліканових глікозилтрансфераз, на кшталт RodA.

Ключові слова: моеноміцин, стійкість до антибіотиків, пептидоглікан.